

Discussion and Summary Remarks*

Bioassays for Carcinogenicity

Much of this conference has concerned itself with a single chemical, di(2-ethylhexyl) phthalate (DEHP). The National Cancer Institute (NCI)/National Toxicology Program (NTP) Bioassay Program, however, has tested a total of five phthalate esters or related compounds for carcinogenic potential. These are reviewed briefly below.

Phthalamide

Phthalamide (NCI TR-161) was found to be of low acute toxicity, allowing it to be tested in mice and rats at doses of up to 3% (by weight) in the diet. Toxic lesions were produced in the livers and urinary tracts of both species, but there was no evidence that phthalamide was carcinogenic. Considerable numbers of animals were diagnosed as having hyperplasia of the urinary bladder, and two of the high-dose female rats had transitional cell carcinomas of the urinary bladder. The report mentions excess calculus formation in the urine, but it is not clear whether that represents precipitated test substance, mineral deposit, or inflammatory exudate that may have been inspissated. Hyperplasia of the bladder mucosa may have been related to the presence of foreign material. On the other hand, it may also have represented focal hyperplasia with dysplasia related to compound administration. A possible limitation in this report is that the control groups are rather small with only 20 animals per group. The test would have been more sensitive with larger control groups. Testicular atrophy cannot be adequately assessed in chronic studies with the Fischer 344 rat because this strain develops a virtually 100% incidence of interstitial cell tumors

in the second year of life, accompanied by germinal cell atrophy. Testicular effects could be addressed with early sacrifices or, better yet, with a different strain of rats.

Phthalic Anhydride

Phthalic anhydride (NCI TR-159) was administered in the diet at doses of up to 5%, and even this level did not appear to be a maximum tolerated dose in the female rats. The test diets were formulated every one or one-and-a-half weeks, even though as much as 25% of the test compound was shown to decompose upon storage for 10 days. The exact doses administered, therefore, are unknown. The only possible evidence for carcinogenicity from phthalic anhydride in this bioassay was the occurrence of excess leukemia in the female rats in the low-dose group. This was interpreted by the authors as not being significant because an excess of leukemia was not found in the corresponding high-dose group. It should be noted, however, that of all the possible cancer end points in the Fischer 344 rat, leukemia is the most variable both in incidence and in time to onset. Additional experimentation with larger groups of animals may be required to evaluate the possible leukemogenicity of phthalic anhydride.

Di(2-ethylhexyl) Phthalate

The report on di(2-ethylhexyl) phthalate (DEHP) has already been reviewed in this conference at some length. An unanswered question in this bioassay is whether palatability or toxicity was the cause of decreased weight gains. Interstitial cell tumors of the testis in male rats were decreased in animals administered the higher dose of DEHP. In contrast to the control group, which had 96% testicular tumors, those administered the high dose of DEHP had only 23% testicular tumors. The rats administered the high dose weighed less and may have had a lower tumor background incidence related to body weight. It is more likely, however, that DEHP had an inhibitory effect on testicular tumor formation,

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and this finding deserves further study. The weight of the evidence for the carcinogenicity of DEHP is very strong. The excess of liver tumors in both sexes of both species in a dose-related manner, with supporting data from *in vitro* transformation experiments, is conclusive.

Di(2-ethylhexyl) Adipate

The bioassay report on di(2-ethylhexyl) adipate (DEHA) indicated that the test chemical produced liver tumors in female mice and possibly in male mice. Not emphasized in the report were early deaths in male mice administered the low dose of the test substance. In the first year of the experiment 22% of this group of animals died from unexplained causes, thus limiting the number of animals at risk to late developing tumors. Liver tumors were found as early as 37 weeks after the beginning of test compound administration, however, suggesting that further evaluation, including age adjusted analyses, may make it possible to reach a more definitive conclusion.

Butyl Benzyl Phthalate

The fifth bioassay conducted by the NCI/NTP was that of butyl benzyl phthalate. The authors of this report concluded that butyl benzyl phthalate may have produced leukemias in female Fischer 344 rats, as they occurred more frequently in the treated animals than in controls. The tumors were of the myelomonocytic type commonly found in the Fischer rat, however, and the background incidence of this tumor type in Fischer rats has been found to be variable. Another experiment, with larger groups of animals, may indicate whether or not butyl benzyl phthalate is capable of producing leukemia in the female Fischer 344 rat. Both testicular and thymic atrophy were reported in butyl benzyl phthalate-treated rats. Testicular atrophy secondary to phthalate ester administration has previously been noted, but the finding of thymic atrophy indicates that immunotoxicological studies should also be considered. The NTP has indicated that the apparent hemorrhage-induced deaths produced in male rats by butyl benzyl phthalate will be explored in another chronic study, with attention given to both disorders of the clotting mechanism of the blood vessels as well as to carcinogenic potential.

Critique

The NCI/NTP Bioassay Program, including the testing of phthalate esters and related compounds, has proved invaluable in identifying chemicals with carcinogenic potential. Review of the technical

reports, however (many of which were, admittedly, only in draft form), indicates that improvement could be made in their contents. More specifically, tabular data need to be rigorously checked for accuracy, experimental findings should be described more completely and the discussion sections ought to be expanded greatly to provide readers with a wider perspective on the chemical under test and explanations for the effects produced.

The descriptive studies of the NCI/NTP have identified a potential problem, the carcinogenicity of phthalate esters. Chronic bioassays, however, do not elucidate the molecular mechanisms of toxicity that are needed to provide credible bases for estimating human risk. Future studies, therefore, should be directed towards the mechanistic aspects of the biological effects of DEHP. A determination of whether DEHP exerts any genotoxic effect or directly damages DNA is a logical starting point. This appears to be an important issue in relation to contradictory results which were obtained in short-term tests in bacteria. It would be very useful to know whether these agents bind covalently to DNA (and, if so, what kind of binding occurs), and whether the radioactivity incorporated into DNA depends upon metabolic activation. This is important not only in the understanding of how these agents may act, but also in interpreting results from the various short-term tests. The simple addition of various types of short-term tests to a study does not necessarily improve the quality of the data that is obtained, nor does it help greatly in interpreting the possible risk of the agent, especially if the series of short-term tests is based upon the same end point. Thus, there is a risk that by adding a lot of information on short-term tests without having parallel studies on the mechanism of action, more confusion than help will be created. A balance between the two approaches appears to be essential.

In vitro cell transformation, on the other hand, may or may not be the result of a DNA damaging event. It would be interesting to determine whether cell transformation in culture is paralleled by increased peroxisome proliferation with this type of agent or with the hypolipidemic drugs. Many carcinogens induce DNA damage and this damage has been causally associated with carcinogenesis. At the same time, however, it should be noted that some carcinogens do not necessarily act through this mechanism. For example, diethylstilbestrol (DES) is carcinogenic in humans and causes cell transformation, but there is only very weak evidence that it binds to DNA, and it is not mutagenic (1, 2). Another example is TCDD, which has been examined for its capacity to bind to DNA by Poland and Glover (3), who showed that none existed. Yet this chemical was a hepatocarcinogen for rats. From the public

health point of view, this type of agent which induces tumors apparently through a mechanism not involving DNA damage should not be considered less dangerous than DNA damaging agents.

Despite these potential problems in the interpretation of mechanistic studies, it is generally felt that such information can provide a more technically sound and scientifically relevant basis for risk assessment than the purely descriptive studies. The bioassay studies, therefore, can be viewed as providing clues to the understanding of the toxic potential of chronic exposure to phthalate esters.

The technical difficulties and high costs involved in mechanistic studies preclude the performance and duplication of each and every possible experiment. It is apparent from this conference, however, that several investigative groups throughout the world are currently engaged in the safety evaluation of phthalate esters. Every attempt should be made to keep all of these groups in close communication regarding the outcomes of the experimental work.*

Short-Term Tests and Their Role in Assessing the Toxic Potential of Phthalate Esters

The Chemical Manufacturers Association (CMA) has proposed a battery of short-term tests to be used in determining which additional phthalate esters ought to be tested for carcinogenicity, and the NTP has outlined a program in progress concerning short-term testing of these compounds. If short-term tests are to be used as predictors of carcinogenicity, then those to be considered should be point mutations in mammalian cells, point mutations in microbial cells, and *in vitro* transformation. This was the grouping of tests suggested by the Interagency Testing Committee some two years ago. Unscheduled DNA synthesis in mammalian cells can be used as an augmenting or supporting assay. However, results from this test system are dependent upon biological effects such as mutation or *in vitro* transformation for interpretation. If short-term tests are to be used to detect mutagenic potential leading to an evaluation of genetic risk, then chromosomal-level assays, such as *in vivo* and

in vitro cytogenetics analysis, would have to be included.

Positive results in the first grouping of assays would suggest a need to assess carcinogenicity in rodents, while positive results in the second grouping would suggest a need to measure germ cell effects in animals. The major application of short-term tests identified by this conference is prediction of carcinogenic potential. At the present time, published results of short-term tests do not generally indicate that DEHP is genotoxic. However, test results on the monoester and the ethylhexyl alcohol are suggestive of such, though not confirmed, and the suggestion is that these components may be mutagenic. Reproducible results in different laboratories are needed to confirm these initial findings. It is essential that this scientific process be satisfied before labels of genotoxicity or lack thereof are applied.

Attention should be paid in the short-term tests to the metabolic activation systems used. For almost all classes of chemicals requiring metabolic activation, this is probably the most critical element in this type of assay. There are various types of metabolic activation systems (e.g., freshly isolated liver cells) which are used in addition to an S9 fraction. The metabolism of DEHP in most short-term test systems is unknown. At a minimum, therefore, the diester, the monoester and the alcohol—i.e., DEHP, mono(2-ethylhexyl) phthalate and 2-ethylhexanol—should be subjected to the short-term tests. The activities of other, lesser metabolites existing in higher oxidized states may have to be evaluated on a case-by-case basis.

By using *in vivo* systems, where possible, the entire spectrum of metabolites can be assessed. This brings to consideration other models for evaluating the phthalates. One assay which permits the metabolic activity of the whole animal to be expressed is the testing of urine from chemically treated animals on microbial or mammalian indicator cells. If positive, this model, coupled with appropriate analytical techniques, could identify the active metabolites. Another whole animal model which could be considered is *in vivo* cytogenetics. It may be argued that this assay is insensitive. However, it may give a truer reflection of any perceived hazard at a reasonable dose level. Further, and just as important, there appears to be a strong correlation between chemicals which are positive in mammalian germ cells—in other words, producing chromosomal level effects—and those which are also positive in *in vivo* cytogenetic assays (e.g., bone marrow cells using metaphase analysis). This is an important aspect of genetic toxicology testing which has not been fully appreciated to date. It is suggested, therefore, that compounds be tested in this model

*In response to this need, the National Toxicology Program (NTP) and the United States Environmental Protection Agency (EPA) have organized a clearinghouse for collection and dissemination of information on phthalates. Contact: Ms. Joan Chase, International Clearinghouse on Phthalate Esters, National Toxicology Program, Landow Building, NIH, Bethesda, Md. 20205.

as a means of selection for dominant lethal or heritable translocation assays. Additional consideration should be given to *in vivo/in vitro* testing. Methods are available to expose liver cells *in vivo*, remove them and measure unscheduled DNA synthesis *in vitro*. This would focus, *in vivo*, on the liver, the organ of most concern in the carcinogenesis bioassay of DEHP.

Finally, a note should be made on the data indicating evidence of dominant lethal effects of phthalate esters in rodents. Evaluation of these experiments are complicated by the use of extremely large, toxic doses and inadequate numbers of females and treated male animals. Many of the phthalate esters are known to damage the seminiferous tubular epithelium of the testes. Moreover, the end point in this test—fetal wastage—could result from a direct toxic, nongenetic effect on the germinal cells. The dominant lethal studies bear repeating, therefore, with the inclusion of doses that are not toxic to the testis.

Review of CMA and NTP Testing Programs

The testing program of the CMA (4) is designed to indicate whether additional phthalate esters need to undergo carcinogenicity testing in animals, while that of the NTP (5) is directed more to determining the mechanism of DEHP carcinogenicity and to evaluating the other toxic effects of phthalate esters. The complementary nature of these two programs is purposeful, rather than coincidental, reflecting a determined effort on the part of both government and industry to avoid costly duplication of effort.

Some of the less obvious points of these programs should be noted. First, if the carcinogenic potential of phthalate esters is being questioned on the basis of the DEHP chronic bioassay results, then the short-term tests utilized to prioritize such agents for carcinogenicity testing ought to be those in which DEHP is active. To date there is no consensus as to whether or not DEHP is active (positive) in short-term tests. The CMA and NTP programs wisely propose, therefore, that any battery of short-term tests used to identify phthalate esters for testing in a bioassay include tests in which DEHP is active.

Secondly, chemicals can cause cancer via nongenetic mechanisms, a likely one for DEHP (based on biological effects) is promotion. The NTP program, therefore, includes the testing of DEHP for liver tumor promotional activity. It must be recognized, however, that there is no single, standard test for liver tumor promoters nor is there any definitive indication of the mechanism of tumor promotion in

the experimental systems currently available. The results of the DEHP promoter studies, therefore, no matter how well conducted, are likely to be confusing and controversial. Nonetheless, their performance may suggest a mechanism of DEHP carcinogenicity.

In addition to the CMA and NTP programs, two groups have indicated that they are currently evaluating the abilities of DEHP, mono(2-ethylhexyl) phthalate and 2-ethylhexanol to bind to liver DNA. These studies are most important not only because they may suggest a mechanism of DEHP carcinogenicity, but because they could indicate which metabolites of DEHP are causative of cancer and whether or not species differences in the metabolism of mono(2-ethylhexyl) phthalate preclude the use of rodents as valid models of human response to DEHP.

Finally, mention has been made of possible epidemiological studies of phthalate ester toxicity. While recognizing that negative epidemiological studies are often inconclusive, it is still felt that an attempt should be made to identify persons with high exposures to phthalate esters and to determine whether their health status has been compromised. Medical patients exposed to DEHP via repeated transfusions or hemodialysis are probably not an acceptable group because of numerous confounding health problems. Both cancer and infertility should be considered in any epidemiological evaluation.

Summary Remarks

It is clear from the data presented at this conference and the resulting discussion that the evaluation of chemical safety is a very complex issue. In addition to the science involved, regulatory decisions must take into account economic impacts, technical capabilities and comparative toxicities of substitutes when the safety of a chemical or a process is questioned. This conference may not simplify the decision-making burden in the case of plasticizers, but public presentation and discussion of the relevant information at least assures that the scientific aspects of the problem have been identified.

The carcinogenesis bioassay of DEHP conducted by the NCI/NTP has been discussed previously in a public forum and was reviewed and approved by a peer review group of independent scientists.* Clearly, DEHP is carcinogenic to rodents. It is recognized, however, that the evaluation of the risk to

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man from exposure to phthalate esters involves more than just a descriptive bioassay. Hence, the need for programs concerning mechanisms of action, pharmacokinetics, interspecies comparisons and epidemiology as discussed at this conference. These efforts are justified, particularly in the case of a chemical which is so widely used and for which there would appear to be no obvious safe substitute.

The limited resources available for chemical safety testing necessitate a mechanism for prioritizing phthalate esters for chronic toxicity studies. Short-term tests may be helpful in this function, but their apparent inability to clearly identify DEHP as a potential carcinogen, with the exception of the recent Japanese studies, is troublesome. It may become necessary to use a different strategy to determine which phthalate esters are in need of assay for carcinogenic potential. The point has been raised at this conference of the need to determine whether DEHP caused tumors in rats and mice by a genetic or a nongenetic mechanism. This information would be helpful in understanding the toxicology of DEHP and in interpreting short-term test results, but the relative safeties of nongenotoxic carcinogens (such as promoters) in comparison to genotoxic carcinogens is not well understood. Simple labeling of chemicals as genotoxic or nongenetic carcinogens, therefore, is not sufficient for safety evaluation.

The emphasis at this conference has clearly been on the carcinogenic potential of phthalate esters. Other toxic effects, such as teratogenicity and infertility, however, should not be ignored in assessing the risk of human exposures to plasticizers. Groups such as the NTP have recognized the importance of nontumor toxic effects and increasingly are incorporating more diverse experimental protocols into their toxicological analyses. Too often the lack of studies designed to evaluate toxicity have been

misinterpreted as a demonstrated lack of toxicity and we must be careful not to repeat such errors, particularly when comparing potential substitutes for phthalate esters.

It is interesting to note that in 1972 the National Institute of Environmental Health Sciences sponsored a Conference on Phthalic Acid Esters (6). Although carcinogenic effects were not a major issue at that time, it was felt nevertheless that the high volume of production of these chemicals and the large number of people exposed to them necessitated a clear understanding of their toxic potentials. We still find ourselves in that situation, needing more information, but with a better idea of what we are seeking. Additionally, the spirit of cooperation shown at this conference between government, industrial and independent scientists suggests that we will reach that goal with all due speed.

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